

08-22-00

A

Please type a plus (+) inside this box - ☒

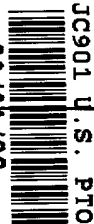
PTO/SB/29 (12/97)

Approved for use through 09/30/00. OMB 0651-0032

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

08/21/00

**UTILITY
PATENT APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.

10583-005-999

Total Pages

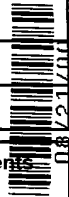
First Named Inventor or Application Identifier

Jifan Hu et al.

Express Mail Label No.

EL 451 594 825 US

PTO/SB/29 (12/97)

**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form
Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 35]
(preferred arrangement set forth below)
-Descriptive title of the Invention
-Cross Reference to Related Applications
-Statement Regarding Fed sponsored R&D
-Reference to Microfiche Appendix
-Background of the Invention
-Brief Summary of the Invention
-Brief Description of the Drawings (if filed)
-Detailed Description of the Invention (including drawings, if filed)
-Claim(s)
-Abstract of the Disclosure
3. ☒ Drawing(s) (35 USC 113) [Total Sheets 10]
4. ☒ Oath or Declaration (Unexecuted) [Total Sheets 2]
a. ☐ Newly executed (original or copy)
b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
i. ☐ **DELETION OF INVENTORS(S)**
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).
5. ☐ Incorporation By Reference (useable if Box 4b is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)
a. ☐ Computer Readable Copy
b. ☐ Paper Copy (identical to computer copy)
c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)
14. ☐ Small Entity ☐ Statement filed in prior application,
Statement(s) Status still proper and desired
15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)
16. ☐ Other:

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

- ☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: filed .

18. CORRESPONDENCE ADDRESS☒ Customer Number or Bar Code Label

24341

(Insert Customer No. or Attach bar code label here)

or ☐ Correspondence address below

NAME				
ADDRESS				
CITY	STATE	ZIP CODE		
COUNTRY	TELEPHONE	FAX		

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

The PTO did not receive the following
listed item(s) Post Card

PENNIE & EDMONDS LLP
COUNSELLORS AT LAW
 1155 Avenue of the Americas
 New York, N.Y. 10036-2711
 (212) 790-9090

ATTORNEY DOCKET NO. 10583-005-999Date: August 21, 2000

Assistant Commissioner for Patents
 Box PATENT APPLICATION
 Washington, D.C. 20231

Sir:

The following utility patent application is enclosed for filing:

Applicant(s): Jifan Hu and Andrew R. Hoffman

Executed on: Unexecuted


Title of Invention: GENE INACTIVATION BY TARGETED DNA METHYLATION

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	34	-20	14	\$18.00 each	\$ 252.00
Independent	1	-3	0	\$78.00 each	\$ 0.00
Minimum Fee					\$ 690.00
Multiple Dependency Fee If Applicable (\$260.00)					\$ 0.00
Total					\$ 942.00
50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern					- \$ 471.00
Total Filing Fee					\$ 471.00

- ☒ Priority of Application No. 60/196,749 filed on April 12, 2000 and Application No. 60/214,148 filed on June 26, 2000 is claimed under 35 U.S.C. § 119.
- ☐ The certified copy of the priority application has been filed in application no. filed
- ☐ Amend the specification by inserting before the first line the following sentence: This is a continuation-in-part of application no. filed .

Respectfully submitted,



Rahul Pathak

42,983

(Reg. No.)

For: Thomas E. Friebe (Reg. No. 29,258)
PENNIE & EDMONDS LLP

Enclosure

This form is not for use with continuation, divisional, re-issue, design or plant patent applications.

GENE INACTIVATION BY TARGETED DNA METHYLATION

RELATED APPLICATION DATA

This application claims the benefit of provisional application Serial
5 No. 60/196,749, filed April 12, 2000, and provisional application Serial No. 60/214,148, filed
June 26, 2000, the entire disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates generally to compositions and methods for the targeted
methylation of DNA. More particularly, the invention relates to compositions and methods for
10 inducing methylation at a target nucleotide sequence located, for example, in a gene or gene
regulatory region. Compositions of the invention include a polynucleotide containing 5-methyl
deoxycytidine (m5C) residues that is capable of inducing methylation at a target nucleotide
sequence.

BACKGROUND

15 The expression of genetic information is central to many biological processes
including disease processes. Genetic information is expressed through the processes of
transcription of a gene (DNA) to form a messenger RNA (mRNA) copy and translation of the
messenger RNA to form a protein. The control of gene expression is disrupted in a variety of
diseases, cancer, in particular. For instance, the *Igf2* gene, encoding an embryonic insulin-like
20 growth factor, is overexpressed in many human tumors, including hepatomas. The increased
expression of this growth factor enhances tumor growth through an autocrine and/or paracrine
mechanism. *In situ* hybridization has demonstrated that *Igf2* expression is highly associated with
the degree of malignancy of hepatoma. SV40 transgenic animals that are deficient in this growth
factor have fewer and smaller tumors than control animals. Previous results in hepatitis B virus
25 (HBV) transgenic mice have shown that *Igf2* correlates with HBV expression, HBV induced
liver injury and liver tumor (Hu, J.F. *et al.* (1997) *Oncogene* 15:2795-2801). Thus, *Igf2* is a
potent mitogen, directly and indirectly involved in the development and progression of many
tumors, especially hepatoma.

Because of the role of inappropriate expression of genes in disease, methods have been sought for inhibiting gene expression. Traditional pharmaceuticals typically target the expressed protein products, and the development of such pharmaceuticals typically requires the laborious screening of a large number of compounds in multiple biological assays. By contrast, inhibition of gene expression offers a means of identifying potential therapeutic compounds directly from gene sequence information. One such technique, the antisense approach, inhibits gene expression with oligonucleotides targeting mRNA for the desired gene. Anti-gene techniques, including triple helix formation, have focused on inhibition of the transcription process through interaction with genomic DNA. Neither of these techniques, however, provide long term inhibition of gene expression.

It has been known for some time that gene expression can be regulated by methylation. Many genes, for example, have been shown to be inactivated by hypermethylation. Such genes are not transcribed and are said to be "imprinted" or "silenced." The *Igf2* gene is normally imprinted in tissues, with only the paternal allele being transcribed (De Chiara, T.M. *et al.* (1990) *Nature* 345:78-80). Human *Igf2* is controlled by four upstream promoters (hP1-hP4). The first promoter (hP1) is located just next to the insulin gene and is not subject to genomic imprinting. The remaining three promoters (hP2-hP4) are all monoallelically expressed from the paternal allele (Vu, T.H. and Hoffman, A. (1994) *Nature* 371:714-717). The mouse *Igf2* contains three promoters that are homologues of human hP2-hP4. As expected, all transcripts of the mouse *Igf2* are imprinted (Hu, J. *et al.* (1995) *Mol. Endocrinol.* 9:628-636). It was thus proposed that an imprinting maintenance element (IME) is located between the nonimprinted hP1 and the three hP2-hP4 promoters and controls the allelic expression of *Igf2*.

Because methylation controls gene expression over a long time period, methods for directed DNA methylation have been sought. Holliday (U.S. Pat. No. 5,840,497) discloses a method for silencing genes that involves the use of a single stranded oligonucleotide containing 5-methyl deoxycytidine residues, wherein the oligonucleotide is complementary to a gene of interest. Binding of the methylated oligonucleotide to a complementary sequence induces methylation of the complementary sequence by a nuclear DNA methylase. However, this method

not efficient enough to allow control of gene expression *in vivo*. Accordingly, there remains a need for efficient means of controlling gene expression in cells by targeted methylation.

SUMMARY

The invention provides a polynucleotide useful for inducing methylation at a target nucleotide sequence within a cell. The polynucleotide includes an oligonucleotide imprinting element that has a first strand and a second strand complementary to the first strand. The first strand can include at least one m5CG sequence which is paired with an unmethylated CG sequence on the second strand. Alternatively, the first strand can include at least one m5CN1G sequence paired with an unmethylated CN2G sequence on said second strand, wherein N1 is any nucleotide, and N2 is a nucleotide that pairs with N1. The first strand and the second strand of the imprinting element can be non-covalently linked, for example through base-pairing interactions, or can be covalently linked, for example by one or more nucleotides forming a loop linker. In a preferred embodiment, the linkage is a single thymine residue.

In one embodiment, the first strand includes at least two m5CG sequences, at least two m5CN1G sequences, or at least one of each. For example, the first strand can contain a 5'-m5CGpN1m5CG-3' (SEQ ID NO:aa) sequence, where N1 is any nucleotide. A preferred imprinting element has first and second strands linked to form a "hairpin" structure. An exemplary imprinting element of this type has the sequence of 5'-CGpApCG-T-m5CGpTpm5CG-3' (SEQ ID NO:cc).

The polynucleotide of the invention also includes a single-stranded oligonucleotide guiding element that is complementary to a target nucleotide sequence in, for example, a gene, preferably a gene regulatory region. A polynucleotide that has a guiding element that targets a gene encoding a protein of unknown function can be employed in studies to determine the function of the encoded protein. A polynucleotide with a guiding element that targets a disease gene, such as a cancer gene, can have prophylactic and/or therapeutic applications.

A guiding element according to the invention includes at least one m5CG sequence m5CG or at least one 5CN3G sequence, wherein N3 is any nucleotide, and is generally

from about 8 to about 50 nucleotides in length. In one embodiment, the guiding element is a 22 nucleotide oligomer having the sequence 5'-AGCCm5CGGGm5CT GGGAGGAGTm5C GG-3' (SEQ ID NO:dd), which targets the promoter of the Igf2 gene.

The imprinting element and guiding element are operably linked such that the polynucleotide is capable of inducing methylation at the target nucleotide sequence. The 5' end of the guiding element can be operably linked, for example, to the 3' end of the imprinting element. A preferred polynucleotide (termed "HepKex") that targets the Igf2 promoter has the sequence 5'-CGACGTm5CGTm5C GAGCCm5CGGGm5C TGGGAGGAGT m5CGG-3' (SEQ ID NO:zz).

Another aspect of the invention is a composition including a polynucleotide of the invention and an additional component, such as a component that facilitates entry of the polynucleotide into a cell and or a physiologically acceptable carrier. In one embodiment, the composition can contain a one or more lipids, such as the cationic lipids N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), dioleoyl phosphatidylethanolamine (DOPE) and/or dioleoyl phosphatidylcholine (DOPC).

The invention also provides a method for targeted DNA methylation wherein introduction of a polynucleotide into a cell induces methylation at a target nucleotide sequence in the cell. The method can be used with any methylation-competent cell and is preferably applied to mammalian cells (especially human cells), plant cells, or prokaryotic cells. The method encompasses the introduction of polynucleotides of *in vivo* as well as *ex vivo*.

In one embodiment, useful in research, the target nucleotide sequence is in a gene encoding a protein of unknown function. In this case, the method typically includes determining a phenotypic change associated with methylation at said target nucleotide sequence after introduction of said polynucleotide. In one embodiment, an organism is produced from a cell after introduction of the polynucleotide. Typically, in this case, the target sequence is in a gene or gene regulatory region, and the organism either does not express the gene or expresses the gene at a reduced level compared to a normal organism.

In another embodiment of the method, the target nucleotide sequence is a disease gene, such as a cancer gene, and methylation at the target nucleotide sequence helps prevent or treat the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically illustrates gene inactivation by a m5C methylated oligonucleotide ("GIT drug") containing an imprinting element ("IE") and a guiding element ("GE").

Figure 2 illustrates the methylation of the human Igf2 promoter 4 (hP4) induced by treatment of Hep 3B cells with an exemplary polynucleotide of the invention ("HepKex," see Example 1). Panel A shows a map of the human Igf2 promoter 4 and indicates the locations of HpaII restriction enzyme sites. Panel B shows a Southern blot probed with an Igf2 probe. Lane 1: molecular weight markers; lane 2: genomic DNA from Hep3B cells digested with PvuII; lanes 3 and 4: genomic DNA from PBS-treated Hep3B cells digested with HpaII (a methylation sensitive enzyme); lanes 5 and 6: genomic DNA from HepKex-treated Hep3B cells digested with HpaII; lane 7: genomic DNA from HepKex-treated Hep3B cells digested with PvuII and MspI (a methylation insensitive enzyme). The appearance of a 1084 basepair (bp) band in lanes 5 and 6 (that is not present in lane 7) demonstrates that HepKex treatment induces DNA methylation in Igf2 promoter 4.

Figure 3 demonstrates the inhibition of Igf2 expression by HepKex in human fibroblasts (3A) and in mouse astrocytes (3B), as quantitated by a polymerase chain reaction (PCR) assay, using β -actin and Igf2 genomic DNA as internal controls. In both cell types, Igf2 mRNA, normalized to actin ("Igf2/actin") or Igf2 (Igf2RNA/DNA) genomic DNA was markedly reduced after a 48 hour treatment with 2 μ M HepKex. Treatment with a control oligonucleotide ("CTII001") or phosphate buffered saline (PBS) did not affect Igf2 expression. See Example 1.

Figure 4 demonstrates the inhibition of Igf2 expression by HepKex in Hep 3B (4A) and Hep G2 (4B) liver tumor cells. The reduction in, or disappearance of, a 115 bp band corresponding to Igf2 mRNA relative the 408 bp band corresponding to Igf2 DNA indicates that

treatment with HepKex inhibited Igf2 expression, whereas treatment with the control oligonucleotide CTII001 or PBS did not. See Example 2.

Figure 5 illustrates the cellular distribution of fluorescently labeled HepKex in cultured Hep 3B tumor cells treated with HepKex in the presence (5A and 5B) or absence (5C and 5D) of a lipid carrier. See Example 3.

Figure 6 shows the tissue distribution of radiolabeled HepKex 90 minutes after injection into a 4 week old Balb/C mouse. See Example 4.

Figure 7 demonstrates the inhibition of Igf2 in mice by HepKex as quantitated by a polymerase chain reaction (PCR) assay, using β -actin and Igf2 genomic DNA as internal controls. Igf2 mRNA, normalized to actin ("Igf2/actin") or Igf2 (Igf2RNA/DNA) genomic DNA was markedly reduced after 3 mg/kg doses HepKex. Treatment with a control oligonucleotide ("CTII001") or phosphate buffered saline (PBS) did not affect Igf2 expression. See Example 5.

Figure 8 shows relative Igf2 expression in mice seven days after treatment with a single 3 mg/kg dose of HepKex or PBS. HepKex blocked more than 75% of the Igf2 expression as compared to the PBS control. See Example 5.

Figure 9 demonstrates inhibition of chloramphenicol acetyltransferase (CAT) by co-transfection with GIT oligos, with and without an imprinting element, in human liver cancer cell line Hep 3B.

Figure 10 demonstrates inhibition of chloramphenicol acetyltransferase (CAT) by co-transfection with GIT oligos, with and without 5' methyl C, in human liver cancer cell line Hep 3B.

DETAILED DESCRIPTION

The invention is based on the discovery that targeted DNA methylation can be achieved using a composition including an oligonucleotide guiding element for targeting the composition to a particular gene or gene regulatory region and an imprinting element that induces DNA methylation at a target sequence.

Definitions

The term "polynucleotide" as used herein refers to any heteropolymer of nucleotide monomers joined with suitable internucleoside groups. The term "monomer" refers to any chemical group that can be incorporated within a polynucleotide chain, including natural nucleotides and non-nucleotides capable of being linked through internucleoside linkages. Nucleotide monomers typically include a nucleobase or simply a "base." Polynucleotides of the invention include those having modified bases such as are disclosed, for example, in U.S. Patent No. 6,001,651. The term "polynucleotide" encompasses heteropolymers containing non-nucleotide monomers or monomers having modified bases. In the polynucleotide deoxyribopolynucleotide, the internucleoside groups joining the nucleotides are phosphodiester groups. Other suitable joining groups include, but are not limited to, phosphorothioates, methylphosphonates, phosphorodithioates, carbamates, amides, and sulfones. Polynucleotides of the invention can contain two or more different internucleoside groups.

An "oligonucleotide" or "oligomer" is a polynucleotide with a length of less than about 100 nucleotides.

An "m5CG sequence" is a dinucleotide sequence with a 5' 5-methylcytidine residue and a 3' guanosine. "m5CpG" denotes a dinucleotide having an internucleoside phosphate linkage.

An "m5CN1G sequence" and an "m5CN3G sequence" both refer to a trinucleotide sequence with a 5' 5-methylcytidine residue linked to any nucleoside, which is linked to a 3' guanosine. In "5pCN1pG," both internucleoside linkages are phosphate linkages.

An "unmethylated" CG sequence" or "unmethylated" CN2G" contains cytidine in place of 5-methylcytidine. N2 is a nucleotide that pairs with N1.

Polynucleotides are said to be "complementary" if they are capable of hybridizing with one another sufficiently well and with sufficient specificity, to give the desired effect. In the context of the invention, "hybridization" refers to hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotide

bases. For example, adenine and thymine are complementary bases which pair through the formation of hydrogen bonds. Thus, as used herein, the term "complementary," refers to the capacity for precise pairing between two nucleotides. If a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of another polynucleotide, then the oligonucleotide and the polynucleotide are considered to be complementary to one another at that position. The oligonucleotides and target nucleotide sequences of the invention are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "complementary" is used herein to indicate a sufficient degree of precise pairing such that stable and specific binding occurs between the oligonucleotide and molecule having a complementary nucleotide sequence. It is understood in the art that the sequence of a first oligonucleotide need not be 100% complementary to that of another to hybridize.

An oligonucleotide is said to be "specific for" a target nucleotide sequence when the oligonucleotide binds to the target nucleotide sequence with sufficient affinity to form a complex with the target nucleotide, and there is a sufficient degree of complementarity to avoid significant non-specific binding of the oligonucleotide to a non-target sequence under conditions in which specific binding is desired. In the case of *in vivo* assays or therapeutic applications, oligonucleotides of the invention are selected to minimize non-specific binding under physiological conditions. In the case of *in vitro* assays, oligonucleotides are selected to minimize non-specific binding under the assay conditions.

Imprinting and guiding elements of a polynucleotide are said to be "operably linked" if the imprinting element is able to induce methylation at a target nucleotide sequence complementary to the guiding element. Operably linked elements can be directly adjacent to one another or can be separated by one or more monomers or other elements.

The phrase "methylation at a target nucleotide sequence" refers to methylation of one or more nucleotides in the vicinity of the target nucleotide sequence. Where the target nucleotide sequence is present in a double-stranded polynucleotide, one or more nucleotides on either strand (or both strands) of the polynucleotide can become methylated. Methylation can be monitored by any convenient method for determining the degree of methylation at a target

nucleotide sequence (see, for example, Vu, T.H. *et al.* (2000) *Genomics* 64:132-143, describing bisulfite genomic sequencing, and Hu, J.F. *et al.* (1998) *Molecular Endocrinology* 12:220-232, describing southern blotting). Gene methylation also can be monitored indirectly by assaying expression at a target gene as shown below in Examples 1, 2, 4 and 5. Generally, methylation occurs within about 5 kilobases (kb) of the target nucleotide sequence. In alternative embodiments, methylation occurs within about 2 kb, about 1 kb, or about 500 basepairs (bp) of the target nucleotide sequence.

As used herein, the term “gene” refers to all nucleotide sequences associated with a gene, including coding sequences; non-coding sequences; such as 5’ and 3’ untranslated regions and introns; as well as any other sequences containing elements that regulate transcription of the gene, such as promoter regions.

The phrase “gene regulatory region” refers to regions including nucleotide sequences containing elements that regulate transcription of a gene.

As used herein, the terms “disease” and “disorder” refer to any condition of an organism or that impairs normal physiological functioning.

As used herein, “a disease gene” is any gene whose expression or overexpression correlates with a disease or disorder.

The term “cancer” refers to any disease characterized by uncontrolled cell growth.

The term “normal organism” is used herein to refer to an organism that has not been subjected to the targeted methylation of the invention.

Introducing a polynucleotide into a cell “*in vivo*” refers to introducing the polynucleotide into a cell when it is part of a multicellular organism. As used in this context, the term “*ex vivo*” refers to introducing the polynucleotide into a cell when it is not part of a multicellular organism. The term “*ex vivo*” encompasses introducing a polynucleotide into a cell, e.g., for research applications, as well as introducing a polynucleotide in to a cell that is then delivered to an organism, e.g., for therapeutic applications.

Polynucleotides

In one embodiment, the invention provides a polynucleotide capable of inducing methylation at a target nucleotide sequence. The polynucleotide includes an oligonucleotide imprinting element operably linked to a guiding element.

5

Imprinting Elements

An oligonucleotide imprinting element is a double-stranded oligonucleotide that enhances the efficiency of methylation of a DNA sequence in the vicinity of the imprinting element. Imprinting elements can be derived from naturally imprinted polynucleotide sequences. Although the invention is not limited to a particular mechanism, imprinting elements are
10 believed to act by recruiting a nuclear DNA methyltransferase complex that can methylate nearby DNA sequences. In one embodiment, the imprinting element includes all or part of a gene regulatory region of a gene that is normally imprinted. Such gene regulatory regions generally contain an imprinting maintenance element (IME), the deletion of which prevents the methylation associated with imprinting. Known IMEs include, for example, those from the Igf2
15 promoter (see Hu, J. *et al.* (1995) *Mol. Endocrinol.* 9:628-636; Birger, Y. *et al.* (1999) *Nature* 397:84-88) and a region of chromosome 15q11-q13 associated with Angelman syndrome and Prader-Willi syndrome (see Saitoh, Shinji *et al.* (1996) *Proc. Natl. Acad. Sci USA* 93:7811-7815).

An imprinting element according to the invention is an oligonucleotide duplex
20 having a first strand and a second strand complementary to the first strand. In one embodiment, the imprinting element has at least one m5CG sequence on the first strand paired with an unmethylated CG sequence on the second strand, thus forming a hemi-methylated duplex. In another embodiment, the imprinting element has at least one m5CN1G sequence on the first strand paired with an unmethylated CN2G sequence, wherein N1 is any nucleotide, and N2 is
25 nucleotide that pairs with N1. In preferred embodiments, the internucleoside linkages in the imprinting elements of the invention are phosphate-based, for example, phosphodiester or phosphorothionate groups. Thus, preferably, the m5CG sequence is an m5CpG sequence, the CG sequence is a CpG sequence, the m5CN1G sequence is an m5CpN1pG sequence, and the m5CN2G sequence is an m5CpN2pG sequence.

In one embodiment, the first strand includes two m5CG sequences, and the second strand includes two corresponding unmethylated CG sequences, thus forming a duplex having two hemi-methylated sites. For example, the first strand can include a 5'-m5CGN4m5CG-3' (SEQ ID NO:aa) sequence, where N4 is any nucleotide. In one variation of this embodiment, the first strand can include the sequence 5'-m5CGTm5CG-3' (SEQ ID NO:bb). In another embodiment, the first strand includes two m5CN1G sequences, and the second strand includes two corresponding unmethylated CN2G sequences, also forming a duplex having two hemi-methylated sites. Alternatively, the first strand can include an m5CG sequence and an m5CN1G sequence, with the second strand including an unmethylated CG sequence and an unmethylated CN2G sequence.

The first strand and the second strand of the imprinting element can form a double helical structure through hydrogen bonding, which, in preferred embodiments, is Watson-Crick hydrogen bonding. The stability of the imprinting element double helical structure is related to the length of the first and second strands as well as the presence or absence of a linkage between the strands. The relative stability of a duplex can be assessed by determining the duplex melting temperature. Also, it is possible to predict a duplex stability using algorithms such as those of Owczarzy, R. *et al.* (*Biopolymers* 1997;44(3):217-39).

If desired, the first and second strands of the imprinting element can be linked covalently to further stabilize the imprinting element. The 5' end of the first strand can be linked to the 3' end of the second strand or vice versa. In addition to end-to-end linkage, the two strands can be attached via a linkage internal to either or both of the strands. In an example of end-to-end linkage, the first and second strands can be linked by one or more nucleotides. Such nucleotide(s) can form a loop connecting the first and second strands, so that the imprinting element forms a so-called hairpin structure.

Parameters for the design of hairpin structures are well known and include the length and sequence of the loop. For example, Vallone *et al.* (Vallone, P.M. *et al.* (1999) *Biopolymers* 50:425-42) teach the optimization of tetraloop sequences. The influence of loops on the stability of DNA duplexes has been studied extensively (see, e.g., Senior, M.M. *et al.* (1988) *Proc Natl. Acad. Sci. USA* 85(17):6242-6; Xodo, L.E. (1988) *Polynucleotides Res.*

16(9):3671-91), and thus those skilled in the art can readily design hairpin structures suitable for use in the imprinting elements of the invention.

In one embodiment, the linker joining the first and second strands of the imprinting element is a single nucleotide, for example a single thymine. A preferred imprinting element of this type has the sequence of 5'-CGACG-T-m5CGTm5CG-3' (SEQ ID NO:cc; with the linker thymine shown in bold).

Non-nucleotide linkers also are useful for covalently linking the first and second strands of the imprinting element. The linker can be, for example, an aliphatic linker, joined to one end of the first strand and an end (usually the closest end) of the other strand. Suitable linkers of this type include, for example, those disclosed by Pils, W. and Micura, R. (2000) *Polynucleotides Res.* 28:1859-1863 and by Durand, M. *et al.* (1990) *Polynucleotides Res.* 18(21):6353-9. Polyethylene and polyethylene glycol linkages can also be employed, as can rigid linkers suitable for hairpin formation, including, for example, stilbene linkers (Nelson, J.S. *et al.* (1996) *Biochemistry* 35:5339-44).

As noted above, linkers can connect the first and second strands of the imprinting element at interior positions such that a linker extends from a base or internucleoside unit on one strand to a base or internucleoside unit on the opposing strand. Alkyl linkers, such as those described by Gao *et al.* (1995) *Polynucleotides Res* 23:285-92), for example, can be employed in the imprinting element to join phosphorothioate internucleoside groups on each strand

The first and second strands of an imprinting element also can be linked multiple times to form a macrocyclic structure. Cyclization of oligonucleotides is known to one of skill in the art (see, for example, Kool, E.T. (1996) *Annu. Rev. Biophys. Biomol. Struct.* 25:1-28) and can readily be employed in the imprinting elements of the invention.

Guiding Elements

The imprinting element is operably linked to a single-stranded oligonucleotide guiding element that is complementary to a target nucleotide sequence in a polynucleotide region to be methylated. The guiding element of the invention includes at least one m5CG sequence or

at least one m5CN3G sequence, wherein N3 can be any nucleotide. In a preferred embodiment, a guiding element has multiple m5C nucleotides. In general, any C residue in the guiding element oligonucleotide can be substituted with a m5C residue. In preferred embodiments, the internucleoside groups are phosphate-based, including, for example, phosphodiester or phosphorothionate groups.

A guiding element has any length that allows specific hybridization to the target nucleotide sequence, preferably from about 8 to about 50 nucleotides, more preferably from about 15 to about 30, and most preferably from about 20 to about 25 nucleotides in length. The length of the guiding element is chosen such that the guiding element can stably and specifically recognize the target nucleotide sequence. A guiding element sequence usually has at least about 16 nucleotides in order to recognize a specific site within a genome. However, because of repetition of DNA sequences, it may be preferable to choose a somewhat longer sequence. Also, because the length of the sequence also affects the stability of the complex formed with the target nucleotide sequence, longer sequences are generally preferred.

The guiding element is operably linked to the imprinting element, such that the linked elements induce methylation at a target nucleotide sequence more efficiently than would the guiding element alone. The guiding and imprinting elements can be linked end-to-end or via a linkage internal to one or both elements. In end-to-end linkage, the 5' end of the guiding element can be linked to the 3' end of the imprinting element or vice versa. In one embodiment, the guiding element and the imprinting element are directly linked by an internucleoside group, such as those described above. In another embodiment, the guiding element and the imprinting element are operably linked with one or more monomers, preferably one or more nucleotide monomers. In addition, any of the alternative linkages described above for linking the first and second strands of the imprinting element can be employed to link the guiding element to the imprinting element.

In an exemplary embodiment, the guiding element is a 22 nucleotide oligomer having the sequence 5'-AGCCm5CGGGm5CTGGGAGGAGTm5C GG-3' (SEQ ID NO:dd), which targets an Igf2 promoter. A preferred polynucleotide having a preferred imprinting element linked to this guiding element has the sequence:

5'-CGACGTm5CGTm5CGAGCCm5CGGGm5CTGGGAGGAGT m5CGG-3' (SEQ ID NO:zz). This polynucleotide directs methylation at a target nucleotide sequence in the Igf2 promoter.

- 5 A guiding element can also be targeted to other genes, including pathogenic genes. For a given gene target, several guiding elements can be synthesized with an appropriate imprinting element. The resulting GIT drug candidate can then be easily for inactivation of the gene target using the screening assays described herein or screening assays known to those of skill in the art for the expression of the target gene. Exemplary GE fragments for several exemplary genes include those described in Table 1 below.

TABLE 1

GUIDING ELEMENTS FOR SPECIFIC METHYLATION OF TARGET GENES

<u>Target Gene</u>	<u>Guiding Elements</u>
c-myc	<u>TCG</u> CTA ATC <u>TCC</u> GCC CAC <u>CGG</u> (SEQ ID NO: __)
	ACC <u>GGC</u> CCT TTA TAA <u>TGC</u> GA (SEQ ID NO: __)
	<u>TCC</u> GCC CAC <u>CGG</u> CCC TTT AT (SEQ ID NO: __)
HIV promoter	CAC <u>GTA</u> GCC <u>CGA</u> GAG <u>CTG</u> (SEQ ID NO: __)
	CCC <u>GAG</u> AG <u>C</u> TGC ATC <u>CGG</u> (SEQ ID NO: __)
	<u>GCT</u> GCA TAT AAG <u>CAGC</u> TG (SEQ ID NO: __)
human urokinase plasminogen activator receptor (uPAR)	AGG <u>CGC</u> CCA <u>CGC</u> ATC TGG (SEQ ID NO: __)
	<u>TCG</u> CTC TTT <u>CGC</u> AAA <u>ACG</u> T (SEQ ID NO: __)
	<u>ACG</u> CAT <u>CTG</u> GGG <u>CTG</u> ACT (SEQ ID NO: __)

human vascular endothelial growth factor receptor (flt-1)	GTT ATA AAT <u>C</u> GC CCC <u>C</u> GC (SEQ ID NO: __)
	G <u>C</u> T GGG GAA AGG TTA TAA AT <u>C</u> GC (SEQ ID NO: __)
	ACC CCT TGA <u>C</u> GT CAC <u>C</u> AG (SEQ ID NO: __)
	CTT CAT <u>C</u> GA GGT C <u>C</u> G <u>C</u> GG (SEQ ID NO: __)
human vascular endothelial growth factor receptor (KDR/flk-1)	C <u>C</u> T GCA <u>C</u> TG AGT CCC <u>C</u> GG (SEQ ID NO: __)
	A <u>C</u> G GGA GAG CCC CTC CTC <u>C</u> GC (SEQ ID NO: __)
human β 3 integrin gene	CAC TGT GGG G <u>C</u> G GGC <u>C</u> GGA (SEQ ID NO: __)
	TGC <u>C</u> GTC CCA CCC ACC <u>C</u> G <u>C</u> G (SEQ ID NO: __)
human 12-lipoxygenase	C <u>C</u> G CAG ACC <u>C</u> GGT CCT TTA A (SEQ ID NO: __)
	C <u>C</u> T GGG <u>C</u> GG TCC <u>C</u> GG GCA (SEQ ID NO: __)
human β -amyloid protein precursor (β -APP)	CTC <u>C</u> GT <u>C</u> AG TTT CCT <u>C</u> GG C (SEQ ID NO: __)
	AT <u>C</u> AG <u>C</u> TGA CT <u>C</u> GC <u>C</u> TGG (SEQ ID NO: __)
human vascular epithelial growth factor (VEGF)	TAG <u>C</u> GG GGA GGA T <u>C</u> G <u>C</u> GG A (SEQ ID NO: __)
	TAA AAG T <u>C</u> G G <u>C</u> T GGT AG <u>C</u> GG (SEQ ID NO: __)
human insulin like growth factor 1 (IGF1)	T <u>C</u> T GTG CTC TAG TTT TAA (SEQ ID NO: __)
	C <u>C</u> A G <u>C</u> T GTT TTC <u>C</u> TG TCT (SEQ ID NO: __)
human epidermal growth factor receptor (HER2)	G <u>C</u> T GCT TGA GGA AGT ATA AG (SEQ ID NO: __)
	AGA ATG AAG TTG TGA AG <u>C</u> T (SEQ ID NO: __)

human tumor necrosis factor α (TNF- α)	TGC <u>C</u> GT TCC TCT ATA AAG (SEQ ID NO: __)
	AGG GAC <u>C</u> TG AG <u>C</u> GTC <u>C</u> GG (SEQ ID NO: __)
human tumor necrosis factor β (TNF- β)	T <u>C</u> G CCC <u>C</u> AG GGA CAT ATA AAG (SEQ ID NO: __)
	CAT ATA AAG G <u>C</u> A GTT GTT (SEQ ID NO: __)
	ACC <u>C</u> AG C <u>C</u> A GCA GAC <u>C</u> GCT (SEQ ID NO: __)
human interleukin 4 (IL-4)	T <u>C</u> G GTT TCA GCA ATT TTA (SEQ ID NO: __)
	TAG AGA TAT CTT TGT <u>C</u> AG C (SEQ ID NO: __)
human granulocyte-macrophage colony stimulating factor (GM- CSF)	CT <u>C</u> TGT GTA TTT AAG AGC T (SEQ ID NO: __)
	C <u>C</u> G CCT C <u>C</u> G TGG CAT TTT G (SEQ ID NO: __)
human interleukin 2 (IL-2)	C <u>C</u> A GAG AGA AGA GTA TAA T (SEQ ID NO: __)
human bcl-2	ATA G <u>C</u> T GGA TTA TAA CTC (SEQ ID NO: __)
	T <u>C</u> G TCC AAG AAT GCA AAG (SEQ ID NO: __)
hepatitis B virus (HBV)	CAG CCA TGG AAA <u>C</u> GA TGT (SEQ ID NO: __)
	TGA AG <u>C</u> GAA GTG CAC A <u>C</u> G G (SEQ ID NO: __)
	AGA <u>C</u> GG TGA GAC <u>C</u> G <u>C</u> GTA (SEQ ID NO: __)
	TGC ATG GTG CTG GTG <u>C</u> GC A (SEQ ID NO: __)

cytomagalovirus (CMV)	TGGG <u>C</u> G GTA GGC GTG TAC GG (SEQ ID NO: __)
	A <u>C</u> G GTA AAT GGCC <u>C</u> G C <u>C</u> T G (SEQ ID NO: __)
	G <u>C</u> G TCA ATG GGG <u>C</u> GG AGT (SEQ ID NO: __)
human c-fos	A <u>C</u> G CTT GTT ATA AAA G <u>C</u> A GT (SEQ ID NO: __)
	T <u>C</u> G TAC TCC AAC <u>C</u> GC AT <u>C</u> TG (SEQ ID NO: __)
human raf-1	<u>C</u> CG AGA GTC TTA AT <u>C</u> G <u>C</u> G G (SEQ ID NO: __)
	T <u>C</u> G <u>C</u> G <u>C</u> AGA AT <u>C</u> GGA GGC (SEQ ID NO: __)

The target nucleotide sequence can be in a gene, the expression of which is to be down regulated, and is preferably in a regulatory region of the gene. Any gene in any organism wherein methylation regulates gene expression can be targeted. Thus, the target nucleotide sequence can be one present in a eukaryotic, prokaryotic (e.g., E. coli), or plant gene. In preferred embodiments the target nucleotide sequence is a mammalian gene sequence, more preferably, a sequence present in an mammal having research or commercial value, such as for example a mouse, rat, cat, dog, or monkey or a chicken, pig, sheep, goat, cow, or horse. In a particularly preferred embodiment, the target nucleotide sequence is a human gene sequence.

Exemplary regulatory regions that can be targeted include, for example, promoter regions and enhancer regions. Target nucleotide sequences can be located, for example, near a TATA box, a GC box, or the binding site of a transcription factor, such as Sp1, and the like. Target nucleotide sequences useful in the invention have at least one CpG or CpN5pG sequence, wherein N5 can be any nucleotide. Preferred target nucleotide sequences are in CpG rich regions, such as CpG islands, or CpN5pG rich regions. Generally, several target nucleotide sequences can be tested as described in Examples 1 and 2 to identify a target nucleotide sequence that gives the desired results for a particular application.

A preferred target nucleotide sequence is in gene regulatory region wherein methylation is known to affect the expression of a nearby gene or genes, i.e., a region that is known to be imprinted.

In preferred embodiments, the gene targeted is a disease gene, such as, for example a gene associated with cancer. Numerous genes have been shown to play a role in the etiology of various cancers. "Oncogenes" is term given to those genes whose overexpression or inappropriate expression plays a role in the initiation or progression of cancer. Any oncogene can be targeted for methylation according to the present invention. Exemplary oncogenes include those encoding growth factors or growth factor receptors. In one embodiment, the target nucleotide sequence is in gene that is normally imprinted (hypermethylated) but becomes hypomethylated in a cancer. The embodiment is illustrated herein using the *Igf2* gene, which is hypomethylated in liver cancers. A polynucleotide of the invention designed to target the *Igf2* gene promoter was shown to inhibit expression of the *Igf2* gene (see Examples 1, 2 and 5). Example 6 shows that, in a mouse model of liver cancer, a composition including this polynucleotide reduces tumor growth *in vivo*.

Compositions

The invention also provides compositions including the polynucleotides of the invention and at least one other component, such as a storage solution (e.g., a suitable buffer); a component that facilitates entry of the polynucleotide into a cell; and/or a physiologically acceptable carrier.

Components that facilitate intracellular delivery of polynucleotides are well-known and include, for example, lipids, liposomes, water-oil emulsions, polyethylene imines and dendrimers, any of which can be used in compositions according to the invention. Lipids are among the most widely used components of this type, and any of the available lipids or lipid formulations can be employed with the polynucleotides of the invention. Typically, cationic lipids are preferred. Exemplary, preferred cationic lipids include N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), dioleoyl phosphatidylethanolamine (DOPE), and/or dioleoyl phosphatidylcholine (DOPC).

In another embodiment, compositions according to the invention include a liposomally entrapped polynucleotide. Liposomes can be composed of various types of lipids, phospholipids, and/or surfactants. These components are typically arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing polynucleotides are prepared by known methods, such as, for example, those described in Epstein, et al. (1985) PNAS USA 82:3688-92, and Hwang, et al., (1980) PNAS USA, 77:4030-34. Ordinarily the liposomes in such preparations are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the specific percentage being adjusted to provide the optimal therapy. Useful liposomes can be generated by the reverse-phase evaporation method, using a lipid composition including, for example, phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). If desired, liposomes are extruded through filters of defined pore size to yield liposomes of a particular diameter.

In another embodiment, compositions of the invention include dendrimers complexed to polynucleotides which can be used to transfect cells. Suitable dendrimers include, but are not limited to, "starburst" dendrimers and various dendrimer polycations. Dendrimer polycations are three dimensional, highly ordered oligomeric and/or polymeric compounds typically formed on a core molecule or designated initiator by reiterative reaction sequences adding the oligomers and/or polymers and providing an outer surface that is positively charged. Such dendrimers may be prepared as disclosed in PCT/US83/02052, and U.S. Pat. Nos. 4,507,466, 4,558,120, 4,568,737, 4,587,329, 4,631,337, 4,694,064, 4,713,975, 4,737,550, 4,871,779, 4,857,599.

Dendrimer polycations are preferably non-covalently associated with the polynucleotides of the invention. This permits an easy disassociation or disassembling of the composition once it is delivered into the cell. Typical dendrimer polycations suitable for use herein have a molecular weight ranging from about 2,000 to 1,000,000 Da, and more preferably about 5,000 to 500,000 Da. However, other molecular weights can also be employed. Preferred dendrimer polycations have a hydrodynamic radius of about 11 to 60 Å., and more preferably about 15 to 55 Å. Other sizes, however, are also suitable for use in the invention. Methods for

the preparation and use of dendrimers to introduce polynucleotides into cells *in vivo* are well known to those of skill in the art and described in detail, for example, in U.S. Patent 5,661,025.

Compositions of the invention can be tested for their ability to deliver polynucleotides into cultured cells by any convenient assay, such as the fluorescent assay described below in Example 3.

Compositions of the invention can include a physiologically acceptable carrier, excipient, or stabilizer, such as those described in Remington's Pharmaceutical Sciences (1980) 16th editions, Osol, ed., 1980. A physiologically acceptable carrier, excipient, or stabilizer suitable for use in the invention is non-toxic to cells or recipients at the dosages employed, and can include a buffer (such as a phosphate buffer, citrate buffer, and buffers made from other organic acids), an antioxidant (e.g., ascorbic acid), low-molecular weight (less than about 10 residues) polypeptide, a protein (such as serum albumin, gelatin, and an immunoglobulin), a hydrophilic polymer (such as polyvinylpyrrolidone), an amino acid (such as glycine, glutamine, asparagine, arginine, and lysine), a monosaccharide, a disaccharide, and other carbohydrates (including glucose, mannose, and dextrans), a chelating agent (e.g., ethylenediaminetetraacetic acid [EDTA]), a sugar alcohol (such as mannitol and sorbitol), a salt-forming counterion (e.g., sodium), and/or an anionic surfactant (such as TweenTM, PluronicTM, and PEG). In one embodiment, the physiologically acceptable carrier is an aqueous pH-buffered solution.

For prophylactic or therapeutic use, polypeptides of the invention are formulated in a manner appropriate for the particular indication. U.S. Patent No. 6,001,651 to Bennett *et al.* describes a number of pharmaceutical compositions and formulations suitable for use with an oligonucleotide therapeutic as well as methods of administering such oligonucleotides. In a preferred embodiment, prophylactic or therapeutic compositions of the invention include polynucleotides combined with lipids, as described above.

Compositions of the invention can be stored in any standard form, including, e.g., an aqueous solution or a lyophilized cake. Such compositions are typically sterile when administered to cells or recipients. Sterilization of an aqueous solution is readily accomplished by filtration through a sterile filtration membrane. If the composition is stored in lyophilized form, the composition can be filtered before or after lyophilization and reconstitution.

Methods

The invention includes a method of using the polynucleotides or compositions of the invention for inducing methylation at a target nucleotide sequence in a cell. Because the polynucleotides of the invention can be directed against any target nucleotide sequence, this method has research, diagnostic, prophylactic, and therapeutic applications.

In one embodiment, the method of the invention includes introducing a polynucleotide of the invention into a cell, thereby methylating a target nucleotide sequence, and then determining a phenotypic change associated with the methylation at the target nucleotide sequence. In research applications, for example, the target nucleotide sequence can be in a gene (preferably in the regulatory region) encoding a protein of unknown function. Protein function can be studied in cultured cells or in “knock-out” organisms by methylating the gene of interest and identifying an associated change in phenotype.

Examples 1 and 2 illustrate how a polynucleotide of the invention can be used to methylate the *Igf2* gene in cultured cells. Conventional knock-out organisms, typically knock-out mice, are well known and are produced by substituting a defective gene for the native gene, e.g., by homologous recombination. Knock-out organisms of the invention, by contrast, retain the native gene, which is either not expressed or expressed at a reduced level due to methylation at a target sequence within the gene, preferably within the gene regulatory region. Thus, references herein to “inactivation of a gene” refer to the inhibition of expression of a gene, as opposed to its disruption. The production of knock-out organisms is described below with respect to producing a knock-out animal. However, one skilled in the art can readily apply these teachings to other organisms.

The present invention encompasses knock-out animals that have a particular gene inactivated in one tissue, a plurality of tissues, or all tissues. To produce a knockout animal having a gene inactivated in one tissue, the polynucleotide of the invention is preferably introduced into a progenitor cell for that tissue. To produce a knockout animal having a gene inactivated in a plurality of tissues, it is preferable to introduce the polynucleotide into a pluripotent cell that gives rise to the tissues for which gene inactivation is desired. Generally, it is preferable to produce a knockout animal having a gene inactivated in all cells, which is

conveniently accomplished by introducing the polynucleotide into a totipotent cell. Totipotent cells are capable of giving rise to all cell types of an embryo, including germ cells. Depending on when gene inactivation occurs, chimaeric animals (i.e., those wherein the gene is active in some cells and not in others) can also be produced from totipotent cells.

5 Totipotent embryonic stem cell lines ("ES" cells) have been isolated by culturing cells derived from very young embryos (blastocysts) (Evans, *et al.* (1981) *Nature*, 292: 154-156; Bradley, *et al.* (1984) *Nature*, 309: 255-258; Gossler, *et al.* (1986) *Proc. Natl. Acad. Sci USA* 83:, 9065-9069; and Robertson, *et al.* (1986) *Nature*, 322: 445-448). Such cells are capable, upon incorporation into an embryo, of differentiating into all cell types, including germ cells, and can
10 be employed to generate animals in which the expression of a particular gene is suppressed. Alternatively, a polynucleotide of the invention can be introduced into the nucleus of one cell which can then be transferred to a fertilized egg using the conventional nuclear transfer techniques that have been employed to clone animals.

Any ES cell may be used in accordance with the present invention. It is, however,
15 preferred to use primary isolates of ES cells. Such isolates may be obtained directly from embryos such as the CCE cell line disclosed by Robertson, E.J., In: *Current Communications in Molecular Biology*, Capecchi, M.R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), pp. 39-44), or from the clonal isolation of ES cells from the CCE cell line (Schwartzberg *et al.* (1989) *Science* 212: 799-803). Such clonal isolation can be accomplished according to the
20 method of Robertson (1987) In: *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, Ed., IRL Press, Oxford. The purpose of such clonal propagation is to obtain ES cells that differentiate into an animal with great efficiency. An example of ES cell lines which have been clonally derived from embryos are the ES cell lines, AB1 (*hprt*⁺) or AB2.1 (*hprt*⁻).

25 The ES cells are preferably cultured on stromal cells (such as STO cells (especially SNL76/7 STO cells) and/or primary embryonic G418 R fibroblast cells) as described by Robertson, *supra*. The stromal (and/or fibroblast) cells serve to eliminate the clonal overgrowth of abnormal ES cells. Most preferably, the cells are cultured in the presence of leukocyte inhibitory factor ("lif") (Gough *et al.* (1989) *Reprod. Fertil.*, 1: 281-288; Yamamori *et al.* (1989) *Science*, 246: 1412-1416). Since the gene encoding lif has been cloned (Gough, *et al.*
30

supra.), it is especially preferred to transform stromal cells with this gene, by means known in the art, and to then culture the ES cells on transformed stromal cells that secrete lif into the culture medium.

ES cell lines useful in the invention can be derived from any species (for example, chicken, *etc.*), although cells derived or isolated from mammals such as rodents, rabbits, sheep, goats, pigs, cattle, primates and humans are preferred. Cells derived from rodents (*i.e.* mouse, rat, hamster *etc.*) are particularly preferred.

Once a polynucleotide of the invention has been introduced into a totipotent cell, the cell is implanted into the uterus of a recipient female and allowed to develop. If instead the polynucleotide is introduced into a progenitor cell or a pluripotent cell, the cell is introduced into an embryo of the appropriate stage, which is then implanted.

Polynucleotides and compositions of the invention can also be used for the study of gene methylation and demethylation processes within a cell. For example, a polynucleotide of the invention can be used to methylate a target nucleotide sequence, which can then serve as a substrate in the study of demethylation processes and/or be used for the study of methylation dependent gene regulation.

In a preferred embodiment, polynucleotides or compositions of the invention are administered to an organism for the prophylaxis or treatment of a disease. The organism is preferably an animal, more preferably a mammal, and most preferably a human known or suspected to be at risk for or suffering from a disease. Diseases amenable to treatment with the polynucleotides and compositions of the invention include those in which gene expression is disrupted, e.g., one or more genes are overexpressed or expressed at inappropriate times or in response to inappropriate stimuli. In preferred embodiments, polynucleotides or compositions of the invention are administered to an organism for the prophylaxis or treatment of cancer. The invention has been exemplified herein for liver cancer.

As described in greater detail below, the polynucleotide or composition is introduced, by any convenient technique, into a cell capable of methylating polynucleotide sequences. Most cells are capable of polynucleotide methylation and can therefore be employed in the invention. Exemplary cells include those from the organisms discussed above with respect

to target nucleotide sequences. In preferred embodiments, polynucleotides are introduced into either mammalian, plant, or prokaryotic cells, with human cells being particularly preferred.

Polynucleotides of the invention can be introduced into a cell *in vivo* or *ex vivo*.

A variety of approaches for introducing polynucleotides into cells *in vivo* and *ex vivo* are known and can be employed in the invention. Preferred methods include lipid or liposome-based delivery (WO 96/18372; WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682-691; Rose U.S. Pat No. 5,279,833; WO 91/06309; and Felgner *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413-7414).

Polynucleotides or compositions of the invention can administered directly to an organism for introduction into cells *in vivo*. The considerations for administering the polynucleotides and compositions of the invention are essentially the same as the considerations for administering antisense, triplex, and other oligonucleotide compositions which are capable of modulating expression of genes implicated in viral, fungal and metabolic diseases. Any route normally used for delivering a molecule, such as an antisense oligonucleotide, to blood or tissue cells can be used. Suitable routes of administration include, for example, intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes. Polynucleotides or compositions of the invention can be administered continuously by infusion, by bolus injection, or by other methods appropriate for the particular preparation.

For prophylactic or therapeutic applications, the dose administered to an individual, in the context of the present invention, should be sufficient to effect a beneficial response in the individual over time (i.e., an effective amount). This amount, which will be apparent to the skilled artisan, will depend on the species, age, and weight of the individual, the type of disease to be treated, in some cases the sex of the individual, and other factors which are routinely taken into consideration when treating individuals at risk for, or having, a disease. A beneficial effect is assessed by measuring the effect of the compound on the disease state in the individual. For example, if the disease to be treated is cancer, therapeutic effect can be assessed by measuring the rate of growth or the size of the tumor as shown below in Example 6, or by measuring the production of compounds, such as cytokines, that indicate progression or regression of the tumor.

Dosing is dependent on severity and responsiveness of the disease state to be treated or prevented, with the course of treatment lasting until a beneficial effect is achieved or, in the case of prophylaxis, for as long as required to prevent onset of the disease. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the individual. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies, and repetition rates. Optimum dosages may vary depending on the relative potency of individual polynucleotides and can generally be estimated based on EC₅₀ found to be effective in *in vitro* and *in vivo* animal models. In general, suitable doses range from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the administered polynucleotide in bodily fluids or tissues.

Following successful treatment, it may be desirable to have the individual undergo maintenance therapy to prevent the recurrence of the disease, wherein the polynucleotide is administered in maintenance doses, ranging from 0.01 µg to 100 g per kg of body weight, once or more daily, to once every 20 years.

EXAMPLES

The following materials and methods are common to all of the examples.

An oligonucleotide having the sequence:

5'-CGACGTm5CGTm5CGAGCCm5CGGGm5CTGGGAGGAGT m5CGG-3' ("HepKex"; SEQ ID NO:zz) was designed to target the most proximal promoter of Igf2 (human hP4 and mouse mP3). This oligonucleotide and a control oligonucleotide ("CTII001") having the sequence: 5'-GGTCACGGTCAGGCGTAGATGG-3' (SEQ ID NO:xx) were synthesized as phosphorothionate deoxyoligonucleotides using standard automated phosphoramidite chemistry and were purified by HPLC. (The CTII001 control oligonucleotide had the same nucleotide content as HepKex, but with a randomized sequence.)

More specifically, HepKex was synthesized using phosphorothionate deoxynucleotide precursors, except that a methylated cytidine precursor (5mdC) was used to introduce methylated

cytidines at desired position in the oligonucleotide. HepKex was synthesized as a single stranded oligonucleotide and, after HPLC, was dissolved in aqueous solution. In this solution, the imprinting element portion of HepKex (5'-CGACG-T-m5CGTm5CG-3' SEQ ID NO: ??) self-anneals to form a hairpin structure.

5

Example 1

This example demonstrates that HepKex can inhibit *Igf2* expression in normal fibroblasts and astrocytes.

Figure 3 shows the expression of *Igf2* in human fibroblasts (HSK09) (panel A) and mouse astrocytes (MBR01) (panel B). HSK09 cells, derived from a 56 day-old human embryo, and MBR01 cells, derived from a newborn F1 mouse both express a substantial amount of *Igf2* in culture. Cells were treated with HepKex (2 μ M), CTII001 control oligonucleotide, or phosphate buffered saline (PBS) for 48 hours and total nucleic acid was extracted and reverse transcribed to produce cDNA. *Igf2* expression was quantitated by polymerase chain reaction (PCR) using a primer set that simultaneously amplified genomic DNA and cDNA. *Igf2* expression was quantitated by scanning the PCR product density of *Igf2* and β -actin (control) DNA and cDNA. Figure 3A shows that HepKex treatment inhibited almost all *Igf2* expression in HSK09 cells, and Figure 3B shows similar results for MBR01 cells.

Example 2

This example demonstrates that HepKex inhibits *Igf2* expression in human liver cancer cells. Human liver cancer cell lines, Hep 3B and Hep G2 (both from American Type Culture Collection, Manassas, VA) express high levels of *Igf2* in culture. *Igf2* expression was analyzed as described in Example 1. As shown in Figure 4, treatment with HepKex at a concentration of 2 micromolar, significantly inhibited expression of *Igf2* (115 bp band labeled "Igf2 mRNA") relative to *Igf2* genomic DNA (408 bp band labeled "Igf2 DNA") in both Hep 3B (Figure 4A) and Hep G2 (Figure 4B) cell lines. Control treatments were carried out with either the control oligonucleotide CTII001 or PBS.

The expression of two putative tumor suppressor genes, H19 and Kip2, which are located in the same chromosome region as Igf2, was also measured in this study. Neither H19 nor Kip2 expression was affected by treatment with HepKex.

Example 3

5 In a study designed to demonstrate that HepKex can reach the nuclei of a human liver cancer cell line (Hep 3B), HepKex was fluorescently labeled an Oligonucleotide Phosphate Labeling Kit purchased from Molecular Probes (Eugene, Oregon). The labeled HepKex was added to Hep 3B tumor cells with or without a cationic lipid carrier (LIPOFECTIN™, lipid N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl
10 phosphatidylethanolamine (DOPE)). Figure 5 shows photographs taken with a fluorescence microscope that illustrate HepKex localization in cells treated with HepKex in the presence (5A and 5B) and absence (5C and 5D) of lipid carrier. The results show that HepKex readily entered and localized in the nuclei of Hep 3B cells, regardless of the presence of the lipid carrier.

Example 4

15 This example shows that HepKex can inhibit expression of Igf2 in an animal. HepKex was liposomally encapsulated in HepLipex. HepLipex is a cationic lipid preparation.

The liver specificity of HepLipex encapsulated HepKex was determined using HepKex that had been labeled radioactively. The HepLipex encapsulated, radioactively labeled HepKex was injected intravenously into a Balb/C mouse. After 90 minutes, the mouse was
20 sacrificed, and tissues were collected. HepKex levels in various tissues were determined by scintillation counting. The resultant tissue distribution of the radiolabeled HepKex is shown in Figure 6, which indicates that approximately 75% of the injected HepKex was found in the liver.

Example 5

This example shows that HepKex can inhibit expression of Igf2 in an animal.
25 HepKex was liposomally encapsulated in HepLipex and injected into four-week old Balb/C mice. Mice of this age express a quantifiable amount of Igf2 in the liver. HepKex (3 mg/kg) was injected into the mouse tail vein every other day. Igf2 expression was quantified using a PCR

assay using β -actin and genomic Igf2 DNA as internal controls. Mice were also treated with the control oligonucleotide CTII001 and PBS. After two doses of the treatment, HepKex suppressed Igf2 expression to very low or undetectable levels in these treated animals, as shown in Figure 7.

The duration of HepKex effect on Igf2 expressed was also studied by injecting four-week old Balb/C mice with a single dose (3 mg/kg) of HepKex or PBS, followed by sacrifice on day seven. As shown in Figure 8, seven days after a single injection, HepKex blocked more than 75% of the Igf2 expression as compared to the PBS control. Expression levels were determined by PCR as described above. Because the effect of HepKex was observed after for a relatively long period after treatment, these data indicate that HepKex effectively imprinted the Igf2 gene.

Example 6

This example demonstrates the anti-tumor activity of HepKex in nude mice. Four nude mice (four weeks old) were transplanted with human liver cancer Hep 3B cells in two lobes of the liver. Two mice received PBS as the experimental control. The other two mice received HepKex encapsulated in HepLipex. Mice were injected through the tail vein with a dose of 3 mg/kg HepKex, three times per week. Three weeks after tumor cell transplantation, control mice began to bear tumors that could be seen outside the skin and died two to three weeks later. The HepKex-treated mice had fewer visible tumors at six weeks after transplantation.

Example 7

This example demonstrates that the GE fragment of a GIT significantly enhances the inhibition efficiency of the GIT. Hep 3B cells were transfected with 10 g of plasmid DNA containing Igf2 promoter 2 and the reporter gene, CAT, by using lipofectin transfection kit (Gibco Life Technologies, Gaithersburg, MD). After 4 hours of transfection, cells were washed with PBS and were treated with mIE (5 μ M, 10 μ M), mP2 (5 μ M, 10 μ M), and PBS, respectively, for 48 hrs in MEM supplied with 10% fetal bovine serum. The mIE is an imprinting oligo with both an IE and a GE that targets promoter 2 of Igf2. The mp2 is the same GE that targets promoter 2 of Igf2 without an IE. After 48 hours of treatment, cells were

collected and analyzed for CAT expression by PCR, using endogenous α -actin as quantitation control.

Figure 9 demonstrates that when compared with PBS control, the GE fragment from Igf2 promoter alone (mp2) inhibits expression of the transfected CAT DNA in Hep 3B cells by 17% (5 M) and 46% (10 M). However, when the IE fragment is linked to the GE (mIE), CAT expression was inhibited by 91% and 98% at the same concentrations of the mP2 GE.

Example 8

This example demonstrates that methylation of the cytosine residues of a GIT significantly enhances the inhibition efficiency of the GIT. Hep 3B cells were transfected with 10 μ g of plasmid DNA containing Igf2 promoter 2 and the reporter gene, CAT, by using lipofectin transfection kit (Gibco Life Technologies, Gaithersburg, MD). After 4 hours of transfection, cells were washed with PBS and were treated with mIE (5 μ M, 10 μ M), IE (5 μ M, 10 μ M), and PBS, respectively, for 48 hrs in MEM supplied with 10% fetal bovine serum. The mIE is an imprinting oligo with both an IE and a GE that targets promoter 2 of Igf2. The IE is an unmethylated version of the mIE. After 48 hours of treatment, cells were collected and analyzed for CAT expression by PCR, using endogenous α -actin as quantitation control.

As shown in Figure 10, the GIT oligo that does not contain m5C can not induce DNA methylation in Igf2 promoter region and failed to block CAT expression in Hep 3B cells.

All publications cited herein are hereby incorporated by reference in their entirety.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

CLAIMS

What is claimed is:

1. A polynucleotide capable of inducing methylation at a target nucleotide sequence comprising:
 - 5 an oligonucleotide imprinting element, wherein said imprinting element is double stranded and has a first strand and a second strand complementary to said first strand, said first strand comprising at least one m5CG sequence paired with an unmethylated CG sequence on said second strand or at least one m5CN1G sequence paired with an unmethylated CN2G sequence on said second strand, wherein N1 is any nucleotide, and
10 N2 is a nucleotide that pairs with N1; and

a single stranded oligonucleotide guiding element comprising at least one m5CG or at least one m5CN3G sequence, wherein N3 is any nucleotide and said guiding element is complementary to a target nucleotide sequence; and

wherein said imprinting element and said guiding element are operably linked to form a
15 polynucleotide capable of inducing methylation at said target nucleotide sequence.
 2. The polynucleotide of claim 1 wherein said first strand and said second strand of said imprinting element are linked through a covalent linkage.
 3. The polynucleotide of claim 2 wherein said first strand and said second strand of said imprinting element are linked by one or more nucleotide residues.
 - 20 4. The polynucleotide of claim 3 wherein said first strand and said second strand of said imprinting element are linked by a single thymidine residue.
 5. The polynucleotide of claim 1 wherein said first strand of said imprinting element comprises at least two m5CG sequences, two m5CN1G sequences, or one m5CG sequence and one m5CN1G sequence.

- 10

19. A composition comprising the polynucleotide of claim 1 and one or more additional components.
20. The composition of claim 19 wherein said additional component is one that facilitates entry of the polynucleotide into a cell.
21. The composition of claim 20 wherein said additional component comprises one or more lipids.
22. The composition of claim 21 wherein said one or more lipids comprise the cationic lipids N-[1-(2,3-dioleoyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), dioleoyl phosphatidylethanolamine (DOPE) and/or dioleoyl phosphatidylcholine (DOPC).
23. The composition of claim 19 wherein said additional component is a physiologically acceptable carrier.
24. A method for inducing methylation at a target nucleotide sequence in a cell comprising introducing the polynucleotide of claim 1 into a cell comprising said target nucleotide sequence, thereby inducing methylation at said target nucleotide sequence.
25. The method of claim 24 wherein said cell is a mammalian cell, a plant cell, or a prokaryotic cell.
26. The method of claim 25 wherein said cell is a human cell.
27. The method of claim 24 wherein said polynucleotide is introduced into said cell *in vivo*.
28. The method of claim 24 wherein said polynucleotide is introduced into said cell *ex vivo*.
29. The method of claim 24 wherein said polynucleotide is introduced as a composition comprising a lipid.
30. The method of claim 24 additionally comprising determining a phenotypic change associated with methylation at said target nucleotide sequence after introduction of said polynucleotide.

31. The method of claim 30 wherein said target nucleotide sequence is in a gene encoding a protein of unknown function.

32. The method of claim 24 additionally comprising producing an organism from said cell, wherein said target sequence is in a gene, and the organism either does not express said gene or expresses said gene at a reduced level compared to a normal organism.

33. The method of claim 24 wherein said target nucleotide sequence is in a disease gene.

34. The method of claim 33 wherein said disease is cancer.

ABSTRACT

The invention provides methods and compositions related to polynucleotides that induce methylation at a target nucleotide sequence within a cell. The polynucleotides include an oligonucleotide imprinting element that has a first strand and a second strand complementary to the first strand. The first strand can include at least one m5CG sequence which is paired with an unmethylated CG sequence on the second strand. Alternatively, the first strand can include at least one m5CN1G sequence paired with an unmethylated CN2G sequence on said second strand, wherein N1 is any nucleotide, and N2 is a nucleotide that pairs with N1. The polynucleotides also include a single-stranded oligonucleotide guiding element that is complementary to a target nucleotide sequence. The guiding element includes at least one m5CG sequence m5CG or at least one 5CN3G sequence, wherein N3 is any nucleotide. The imprinting element and guiding element are operably linked such that the polynucleotide is capable of inducing methylation at the target nucleotide sequence.

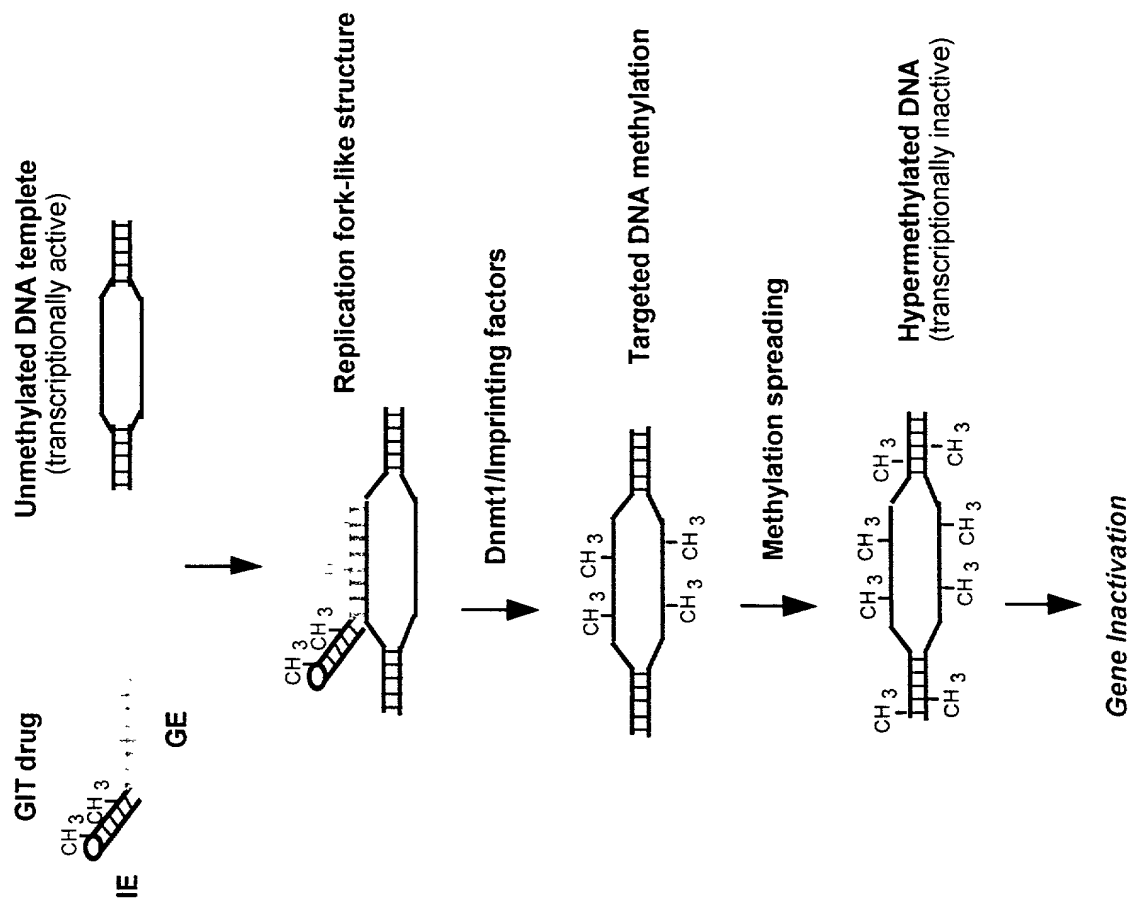


FIGURE 1

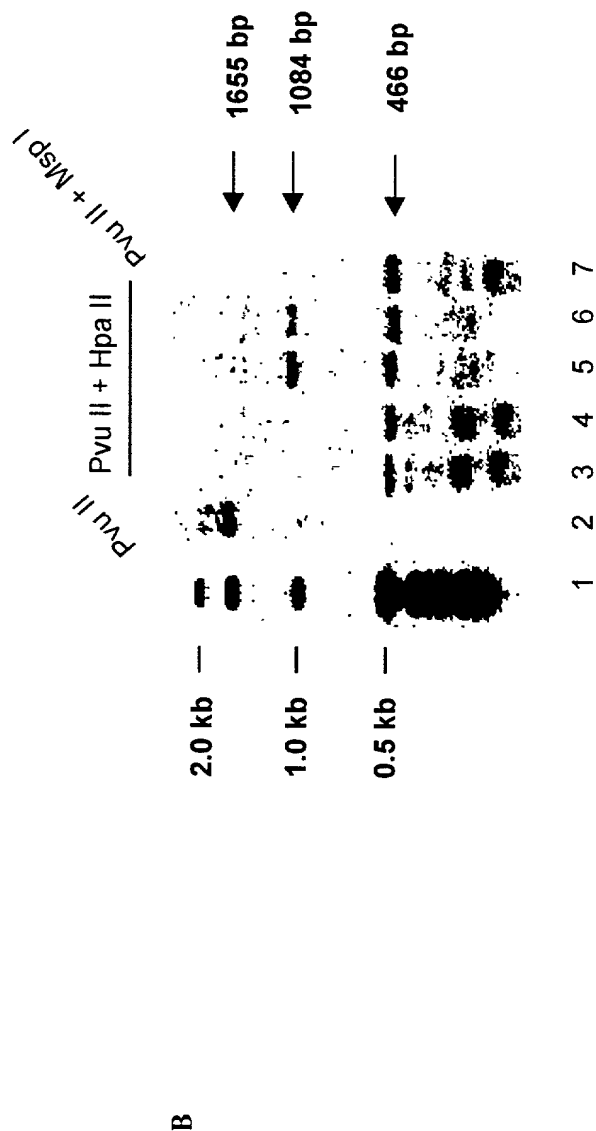
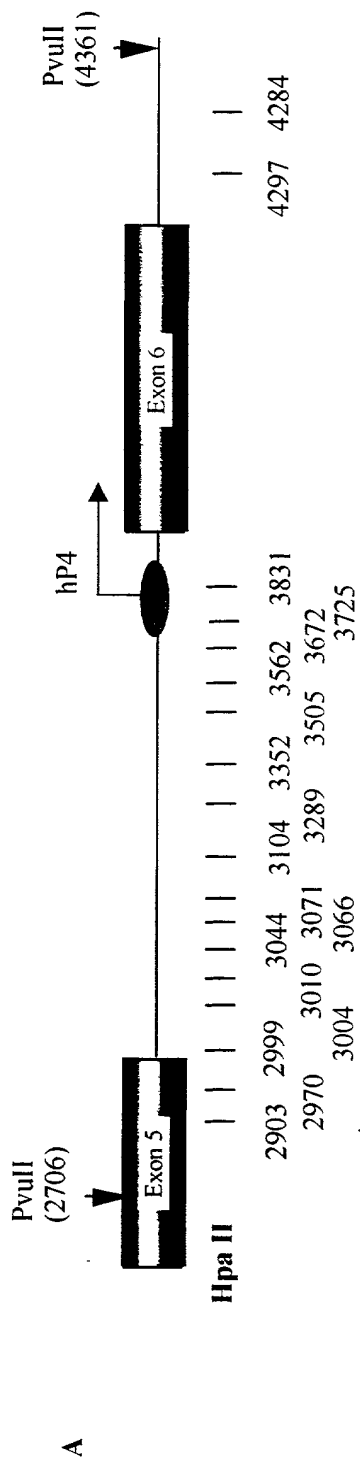


FIGURE 2

FIGURE 3

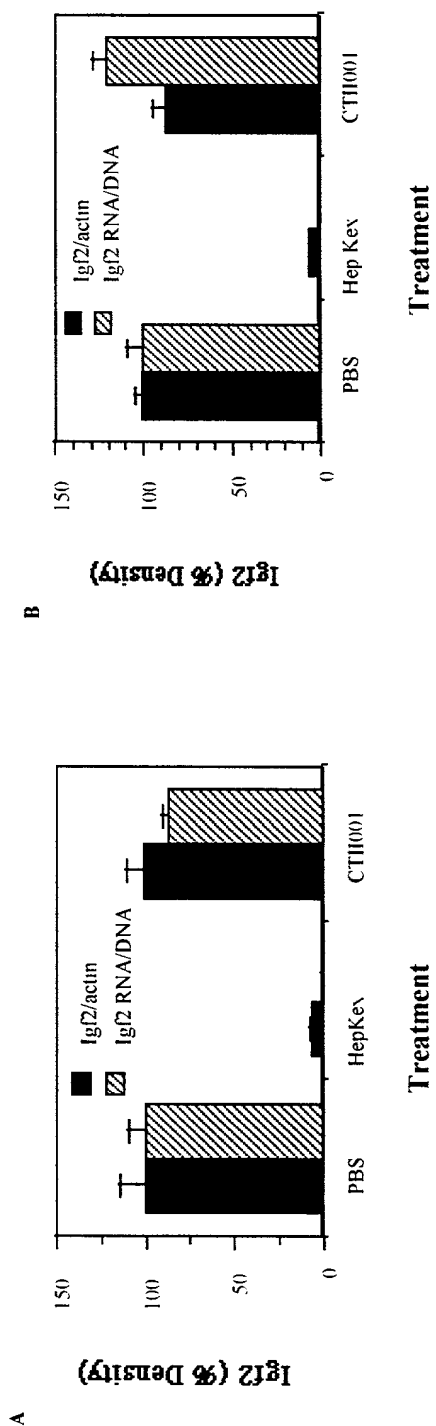
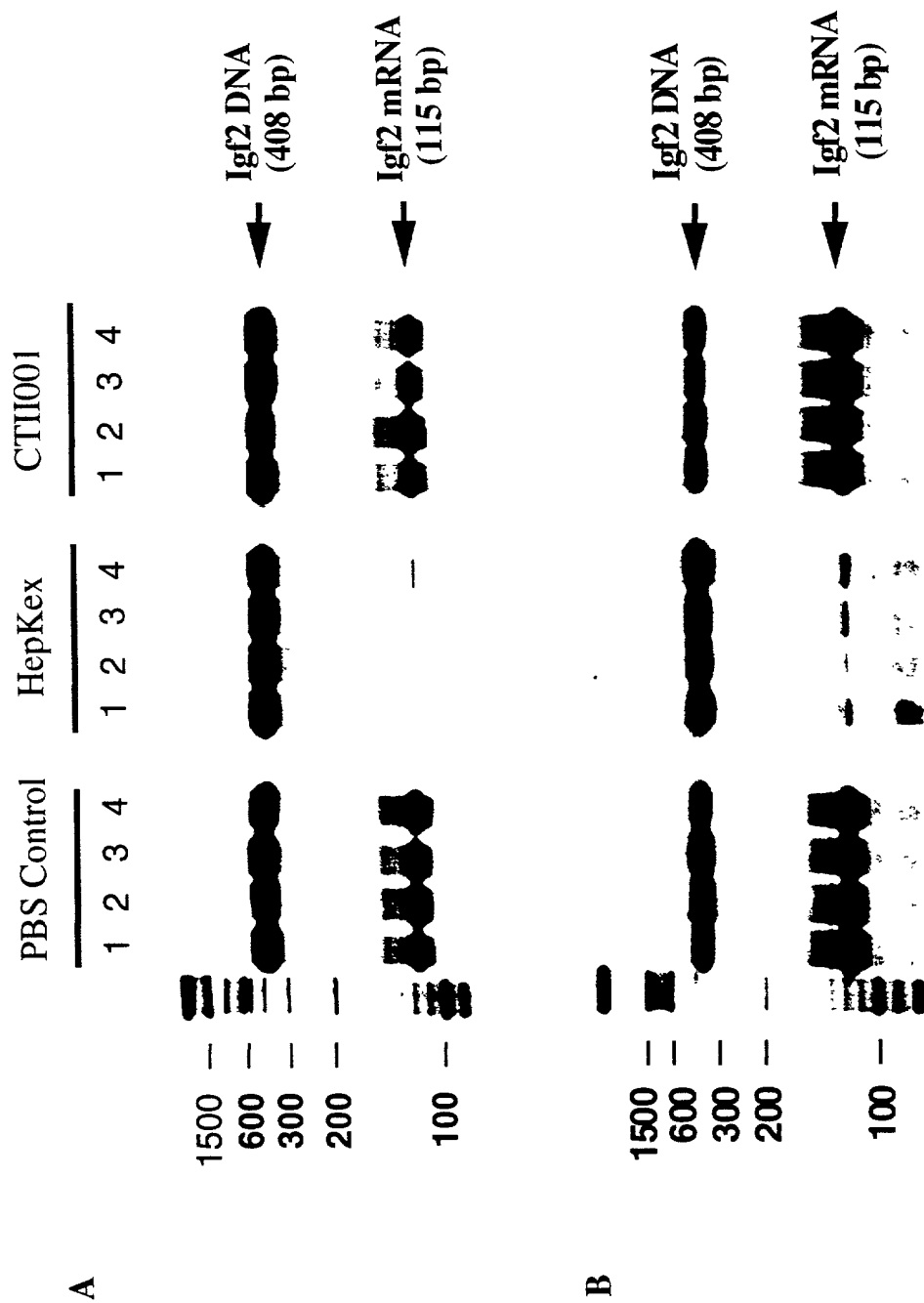


FIGURE 4



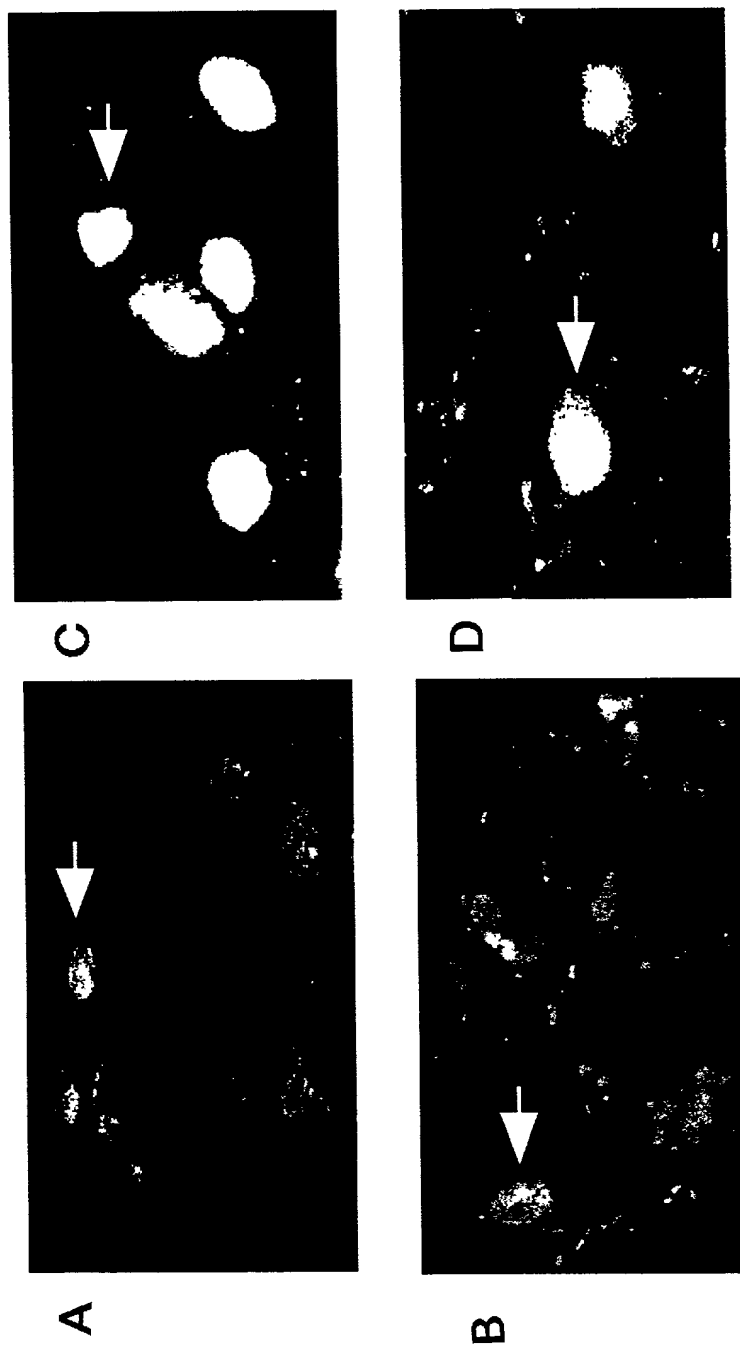


FIGURE 5

096473438, 082400

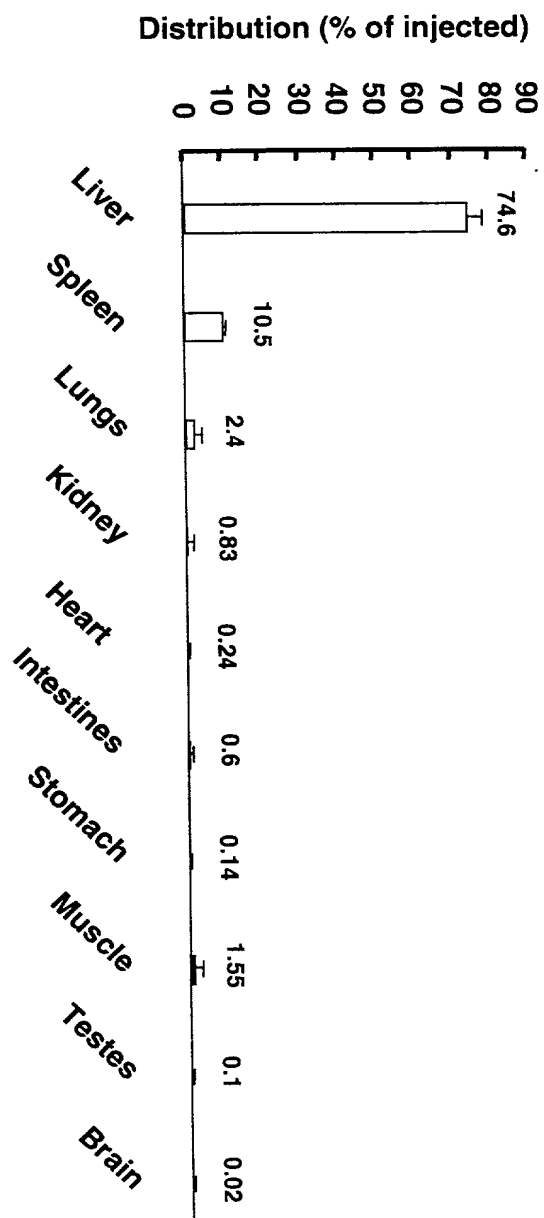


FIGURE 6

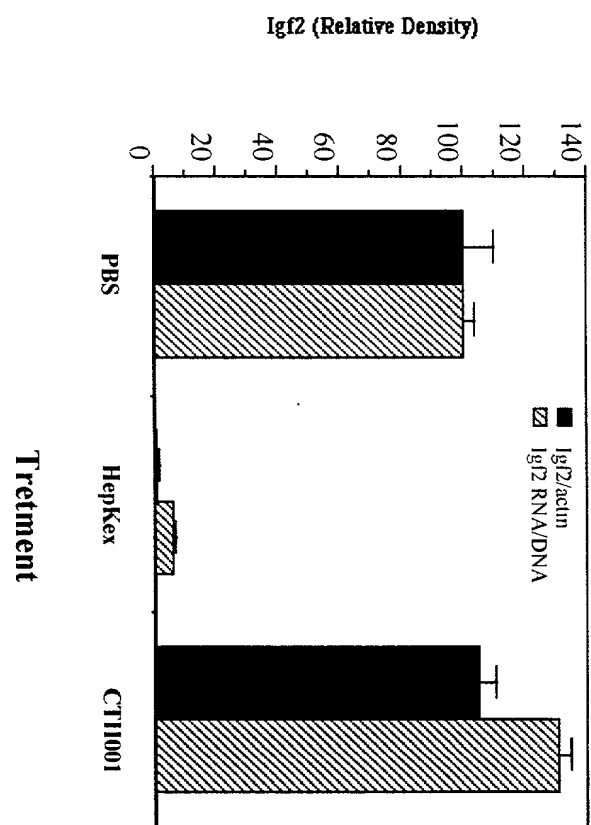


FIGURE 7

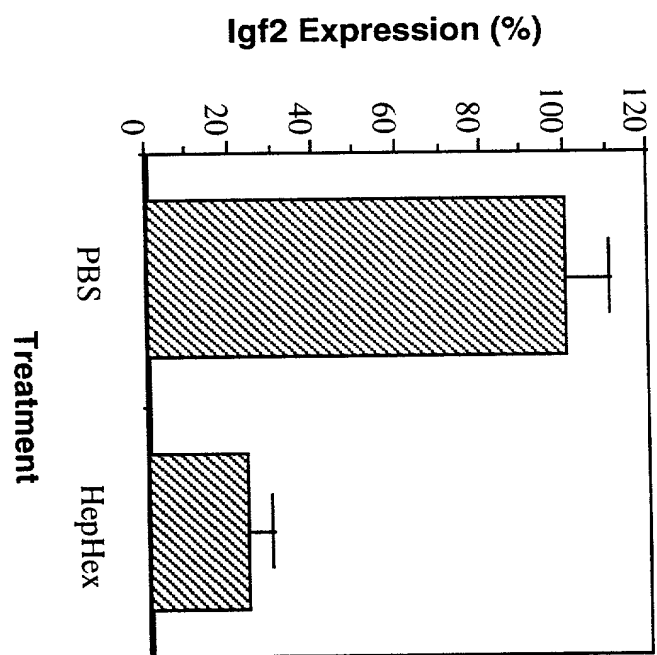


FIGURE 8

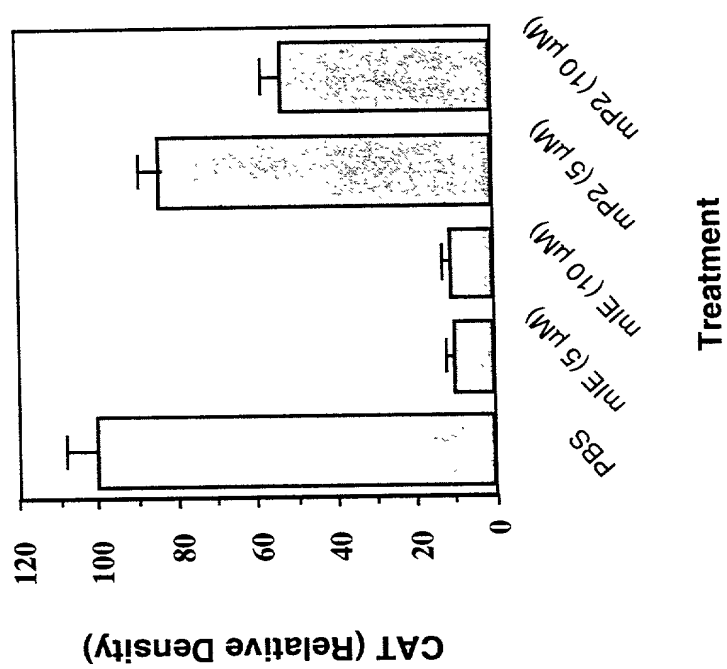


FIGURE 9

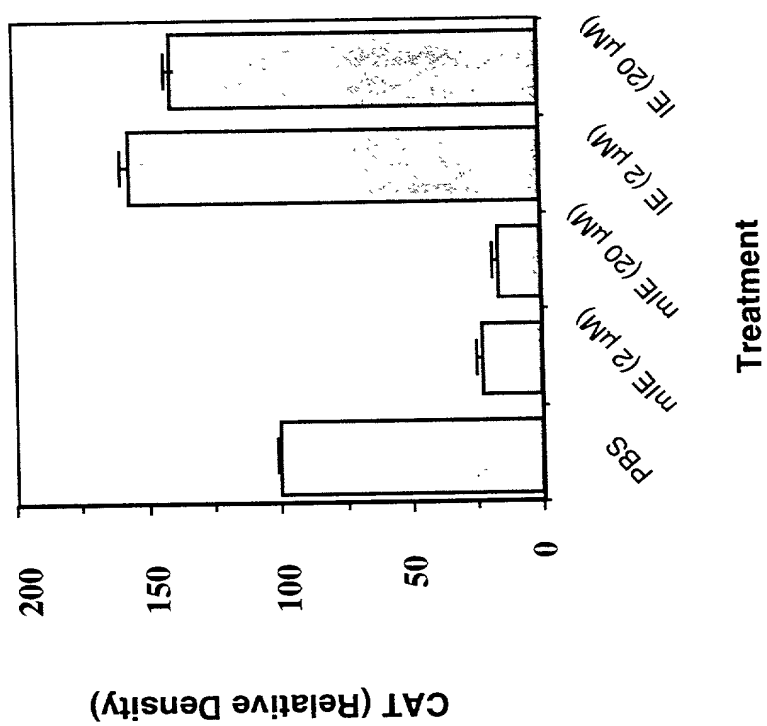


FIGURE 10

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENE INACTIVATION BY TARGETED DNA METHYLATION

and for which a patent application:

- ☒ is attached hereto and includes amendment(s) filed on *(if applicable)*
☐ was filed in the United States on as Application No. *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE
60/196,749	4/12/00
60/214,148	6/26/00

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebe (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Samuel B. Abrams (Reg. No. 30605), Steven I. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bannon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriane M. Antler (Reg. No. 32605), Thomas G. Rowan (Reg. No. 34419), James G. Markey (Reg. No. 31636), Thomas D. Kohler (Reg. No. 32797), Scott D. Stimpson (Reg. No. 33607), Gary S. Williams (Reg. No. 31066), William S. Galliani (Reg. No. 33885), Ann L. Gisolfi (Reg. No. 31956), Todd A. Wagner (Reg. No. 35399), Scott B. Familant (Reg. No. 35514), Kelly D. Talcott (Reg. No. 39582), Francis D. Cerrito (Reg. No. 38100), Anthony M. Insigna (Reg. No. 35203), Brian M. Rothery (Reg. No. 35340), Brian D. Siff (Reg. No. 35679), and Alan Tenenbaum (Reg. No. 34939), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 3300 Hillview Avenue, Palo Alto, CA 94304, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO:

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, N.Y. 10036-2711

DIRECT TELEPHONE CALLS TO:
PENNIE & EDMONDS LLP DOCKETING
(212) 790-2803

201	FULL NAME OF INVENTOR	LAST NAME Hu	FIRST NAME Jifan	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Palo Alto	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 745 Seminole Way	CITY Palo Alto	STATE OR COUNTRY California	ZIP CODE 94303
202	FULL NAME OF INVENTOR	LAST NAME Hoffman	FIRST NAME Andrew	MIDDLE NAME R.	
	RESIDENCE & CITIZENSHIP	CITY Palo Alto	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 1177 Waverly Street	CITY Palo Alto	STATE OR COUNTRY California	ZIP CODE 94301
203	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
204	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
206	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 JIFAN HU	SIGNATURE OF INVENTOR 202 ANDREW R. HOFFMAN	SIGNATURE OF INVENTOR 203
DATE	DATE	DATE
SIGNATURE OF INVENTOR 204	SIGNATURE OF INVENTOR 205	SIGNATURE OF INVENTOR 206
DATE	DATE	DATE